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Original article

Oncolytic Newcastle disease virus reduces growth of cervical cancer cell by inducing apoptosis

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ABSTRACT

Although Oncolytic viruses have been regarded as a promising tool for targeted therapy of cancer, accomplishing high efficacy and specificity with this strategy is challenging. Oncolytic virotherapy is one of the novel therapeutic methods recently used for the therapy of human malignancies. Cervical cancer is on the major public health problem and the second most common cause of cancer death among females in less developed countries. The aim of this study was mainly to determine the apoptosis effect of oncolytic Newcastle disease virus (NDV) in TC-1 cell line.

In the current study, the oncolytic NDV, vaccine strain LaSota, was used to infect murine TC-1 cells of human papillomavirus (HPV)-associated carcinoma which expressing human papillomavirus 16 (HPV-16) E6/E7 antigens in vitro. The effectiveness of NDV for cervical cancer cell line was investigated by evaluating the antitumor activity of oncolytic NDV and the involved mechanisms. Antitumor activities of oncolytic NDV were assessed by cell proliferation (MTT) and lactate dehydrogenase (LDH) release analysis. In addition, molecular changes of early stage of apoptosis and the role of reactive oxygen species (ROS) were analyzed by flow cytometry and Western Blot in NDV-treated TC-1 cells.

The results showed that NDV treatment significantly decreased the viability of a TC-1 cell line and suppressed the growth by inducing apoptotic cell death. In addition, we demonstrated that NDV-induced apoptosis of TC-1 cells is mediated by ROS production. In summary, our findings suggest that oncolytic NDV is a possible therapeutic candidate as a selective antitumor agent for the treatment of cervical cancer.

1. Introduction

Cervical cancer is known as of the most frequent malignancies which can be observed among women all around the globe, with annual 530,000 new cases and 250,000 deaths (Liu et al., 2018, Gableh et al., 2016, Mohebbi et al., 2019). Unusual vaginal bleeding is one of the most common symptom of cervical malignancy in advance stage. The major causes of this cancer include Human papillomaviruse-16 (HPV-16) and HPV-18, being responsible for nearly 70% of all cervical cancers (Khan et al., 2005, Descamps et al., 2009). Viral early proteins E6/E7 play a key role in the progression of this cancer (Park et al., 2016, Baghban Rahimi et al., 2018). Surgery, radiotherapy, chemotherapy, immunotherapy or combination of these methods are common treatments for cervical cancer. Since these currently developed treatments have shown several drawbacks, novel approaches are highly required to efficiently address the therapeutic challenges. Among the possible alternative strategies, oncolytic viruses have been regarded as promising therapeutic candidates for cancer therapy (Zamarin et al., 2014).

One of the characteristics which distinguish the cancer cells from the normal cells is deficiency in Interferon (IFN) pathway. By making the most of this defect, oncolytic viruses can continues

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replication in cancer cells, thereby leading to destruction and induction of oncolysis (Yurchenko et al., 2018). Oncolytic viruses (OVs) are a class of viruses that selectively replicate in malignant cells and subsequently diffuse in a tumor without damaging the normal cells. So far, two oncolytic viruses Onyx-015 (in China) and T-VEC (in USA) were approved for head and neck squamous cell carcinoma (HNSCC) and metastatic melanoma subjects, respectively (Russell and Peng, 2018, Woller et al., 2014). These viruses are classified into two groups: (i) those naturally replicating and damaging the cancer cells with no pathogenicity in humans, and (ii) genetically engineered viruses that are used as vaccine vectors (Jhawar et al., 2017).

Newcastle disease virus (NDV) is a member of naturally oncolytic viruses which belongs to the genus Avulavirus, family Paramyxoviridae and causes severe illness in birds and poultry. Therefore, since this virus is non-pathogenic in mammals, it can be used as an agent for cancer treatment (Schwaiger et al., 2017). NDV categorized into three pathotypes according to chicken pathogenicity, including lentogenic (avirulent), mesogenic (intermediate), and velogenic (virulent). LaSota and Hitchner B1 are lentogenic strains that to be considered as live vaccines against Newcastle disease (Panda et al., 2004). NDV has completed phase I/II clinical trial in patients with glioma, and preclinical studies have been mainly focused on the direct effect of this virus in vivo. A study has demonstrated the effect of NDV on different cancer models such as glioma and melanoma (Koks et al., 2015). However, there is a paucity of information regarding the role of this virus in cervical cancer. For this purpose, we evaluated the oncolytic potential of NDV (Lasota) on TC-1 cells, which is cervical cancer model of C57 mice.

2. Materials and methods

2.1. Cell culture

Mouse TC-1 cells (express HPV-16 E6/7 oncoproteins and purchased from the cell bank of Pasteur Institute, Tehran, Iran) were grown in complete DMEM medium (Gibco, UK) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 25 mM HEPES, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C with 5% CO2.

2.2. NDV purification

The LaSota strain of NDV vaccine was prepared and inoculated into allantoic cavity of embryonated specific pathogens free (SPF) eggs, purchased from Razi Vaccine and Serum Research Institute (Tehran, Iran). After 120 h, the allantoic fluid was collected, followed by centrifuging at 4 °C. In the next step, the supernatant was separated and centrifuged for 3 h at 4 °C (30,000 g/min). The re-suspension of purified virus was carried out in Phosphate-buffered saline (PBS) (pH 7.4), followed by titration via haemagglutination (HA) test. Eventually, obtained virus was stored at −80 °C for further analysis.

2.3. MTT assay

The cytotoxic effects of the NDV was evaluated by Cell Proliferation assay Kit (MTT) (sigma, USA). For this regards, TC-1 cells were seeded in each well of plates at a 3.0×10^4 density and were grown in these cell culture plates for 24 h. After infecting the cells with NDV at Multiplicity of infections (MOIs) of 1, 5, 10, 20 and 40 for 1 h, medium was removed and renewed by DMEM. Following incubation time of 72 h, each well received 100 μl of MTT solution with subsequent incubation at 37 °C for 3 h. After removing incubation medium, 100 μl dimethyl sulfoxide (DMSO) was used to dissolve the purple crystals of formazan. Finally, the absorbance was measured at 540 nm using absorbance microplate reader aiming to assess the MTT reduction (Anthos Labtec Instruments, Austria). Each test was repeated at least three times.

2.4. LDH release measurement

Cell lysis was determined by evaluation the secretion of lactate dehydrogenase (LDH) into culture medium by LDH assay kit (Takara Bio, Tokyo, Japan), with following the protocol of the kit. Briefly speaking, TC-1 cells were placed at a 3.0×10^4 cells/100 μl density in 96-well plates, and were infected with NDV at MOIs of 1, 5, 10, 20 and 40 for 1 h. after that the media (DMEM) was renewed and supplemented by 1% FBS, pen/strep 1% for 72 h, the centrifugation of plate was performed at 250g/min for 10 min. Then, 100 μl of each well's supernatant was added to the wells of a new 96-well plate, followed by adding 100 μl of LDH test solution to each well and room temperature incubation for 30 min. Finally, absorbance was read in 490 nm wavelength and the calculations were done according to the formula contained in the kit. Each test was repeated at least three times.

2.5. Cell apoptotic rate assay

Induction of NDV induced apoptosis of TC-1 tumor cells was demonstrated by flow cytometry based Annexin V/PI staining. The NDV effect on the apoptosis of TC-1 cells was tested via FITC Annexin-V/PI Apoptosis Detection Kit (BD Biosciences, USA). In summary, 5×10^5 cells were seeded in each well of a 6-well plate. Then, TC-1 cells were infected with NDV at MOIs of 1, 10, 20 and 40 for 1 h and media was renewed by DMEM containing 1% pen/strep for 72 h. Uninfected cells were considered as control. Further, the cells were trypsinized and exposed to the medium to neutralize the effect of trypsin. Next, 5 μl of FITC-conjugated anti-annexin-V/PI staining antibody (BD Biosciences, USA) and 5 μl of propidium iodide solution were thoroughly mixed with 100 μl of the binding buffer. Then, the cells were subjected to 15 min incubation at room temperature and in darkness, and the cell apoptotic rate was determined. For flow cytometric Annexin V staining, means of ratio of annexin V stained cells was compared with their corresponding controls.

2.6. Western blot (WB) analysis

The level of caspase 9 in TC-1 cells was determined via infecting them with NDV at a MOI of 20. Following 72 h, TC-1 cells were collected and lyzed with RIPA buffer. Following centrifugation, the supernatant was isolated and filtered through 0.45 μm filter and protein concentration was calculated by Bicinchoninic acid assay (BCA) (Thermo Fisher Scientific). The cell lysates were exposed to 15% Sodium Dodecyl Sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, proteins were loaded onto the polyvinylidene difluoride (PVDF) membrane. Followed by membrane blocking via BSA and overnight exposure to the appropriate primary anti-caspase 9 and anti-β-actin (loading control) antibodies in a temperature of 4 °C (1:1000, abcam, Cambridge, MA, USA). The membrane was then washed and exposed to rabbit anti-mouse secondary antibody (1:10,000, Sigma, St. Louis, MO, USA). Finally, DAB was used to visualize the obtained bands (Sigma). Densitometric assessment of protein bands was done by ImageJ software (NIH, Bethesda, USA), and their ratio to β-actin was calculated.

2.7. Measurement of intracellular ROS

The evaluation of reactive oxygen species (ROS) level was achieved by 2′, 7′-dichlorofluorescein diacetate (DCFH-DA). Concisely, the seeding of TC-1 cells was conducted in a 6-well plate at a density of 5×10^5 cells per well and then infected with MOI of 20 for 1 h. Subsequently, media was renewed by DMEM containing 1% pen/strep for 72 h. After adding DCFH-DA to the samples (for 1 h), they were trypsinized and washed with PBS. Finally, fluorescence intensity of cells was calculated.

2.8. Statistical analysis

All statistical analyses were performed using the Graph Pad Prism version 5.01 (GraphPad Software, CA, USA). A one-way ANOVA (followed by Tukey's post-hoc test) and student's *t*-test were used to compare means among groups. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were considered as values of significance.

3. Results

3.1. The Cytotoxicity effect of NDV using MTT assay

To determine whether oncolytic NDV has cytotoxic effects on TC-1 cells, MTT reduction assay for various MOI of NDV (1–40) was applied. Because the MTT reduction of mitochondrial enzyme can only occur in metabolically active cells, the level of activity is measured as a cell viability. As shown in Fig. 1, NDV effectively reduced the viability of TC-1 cells, which was MOI-dependent. The viability in un-infected cells was considered as control. Our findings also indicated that the viability of the TC-1 cells were declined to 54% and 43% at a MOI of 20 and 40 respectively ($P < 0.001$).

3.2. The cytotoxic effects of NDV using LDH assay

Lactate dehydrogenase (LDH) is a cytosolic enzyme which can be secreted by necrotic cells. Therefore, intracellular LDH can be considered as a biomarker for cytolytic activity of NDV. The cytotoxic effect of NDV was assessed through LDH release after 72 h incubation with different MOI on TC-1cells.

As demonstrated in Fig. 2, the rate of LDH release in NDV-induced TC-1 cells increased in a MOI dependent manner than control cells. The results also showed that maximum release, 49.8% ± 5.6, was observed 72 h after infection at a MOI of 40 in the TC-1 cells ($P < 0.001$). Although TC-1 cultures infected with 40 MOI of NDV did not show statistically significant viability than cells infected with the 20 MOI ($P > 0.05$).

3.3. The effect of the NDV on apoptosis induction using annexin V/PI

Assessment of the percentage of apoptosis/necrosis induction in NDV- treated TC-1 was conducted by using flow cytometry, via annexin V/PI staining. The means of the percentage ratio of Annexin V positive and PI negative (early apoptosis/viable cells) TC-1 cells were plotted for with NDV at different MOIs (Fig. 3).

The results also showed that maximum apoptosis induction (early stage), 6.3% ± 0.32, was observed 72 h after infection at a MOI of 40 in the TC-1 cells ($P < 0.001$). Although TC-1 cultures infected with 40 MOI of NDV did not show statistically significant apoptotic activity than cells infected with the 20 MOI ($P > 0.05$). The results of experiment indicated that TC-1 cells inoculated with a MOI of 20 provided the highest oncolytic titer at 72 h post infection (Zhang et al., 2017). Thus, the MOI of 20 was chosen as optimum dose for subsequent experiments.

3.4. NDV-induced apoptosis in TC-1 cells

In order to assess the expression level of caspase 9 protein in infected and un-infected cells, the cell lysate supernatants were exposed to SDS-PAGE and WB after 72 h. As shown in Fig. 4, a significant increase in caspase 9 protein was detected in TC-1 cells infected by NDV with MOI 20 compared to the control group ($p < 0.01$).

3.5. The impact of the NDV on intracellular levels of ROS

The induction of ROS has been claimed to be the underlying modality for various anti-cancer therapies that trigger apoptotic

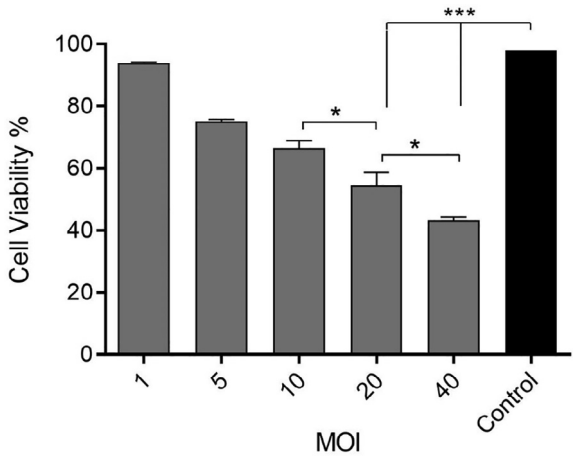

Fig. 1. Cytotoxicity of NDV on TC-1 cells evaluated by MTT assay. TC-1 cells were seeded in 96-well plates and treated with various MOIs of NDV for 72 h. The MTT assay results indicated that infection with 40 MOI can significantly reduce viability TC-1 cells vs control. $^{***}(P < 0.001)$ indicates statistically significant difference between MOIs 40 and 20 compared with control group by one-way ANOVA. $^{*}(P < 0.05)$ indicates statistically significant difference between MOI 20 compared with MOI 40 group by one-way ANOVA.

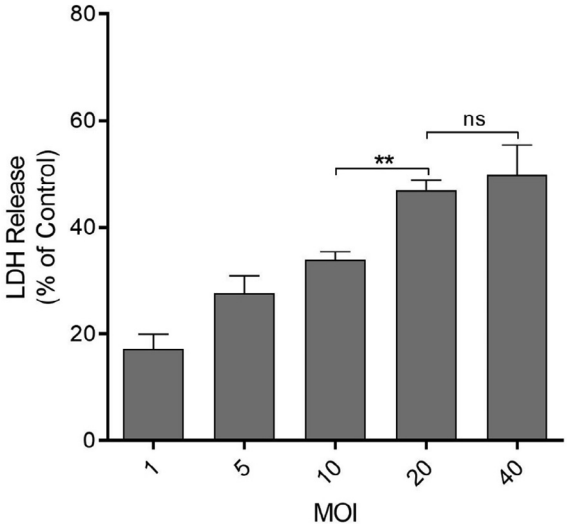

Fig. 2. Cytotoxicity of NDV on TC-1 cells evaluated by LDH assay kit. TC-1 cells were seeded in 96-well plates and treated with various MOIs of NDV for 72 h. The LDH assay results indicated that infection with 20 MOI can significantly release LDH vs control. Although TC-1 cultures infected with 40 MOI of NDV did not show statistically significant viability than cells infected with the 20 MOI ($P > 0.05$). $^{**}(P < 0.01)$ indicates statistically significant difference between MOI 20 compared with MOI 10 group by one-way ANOVA.

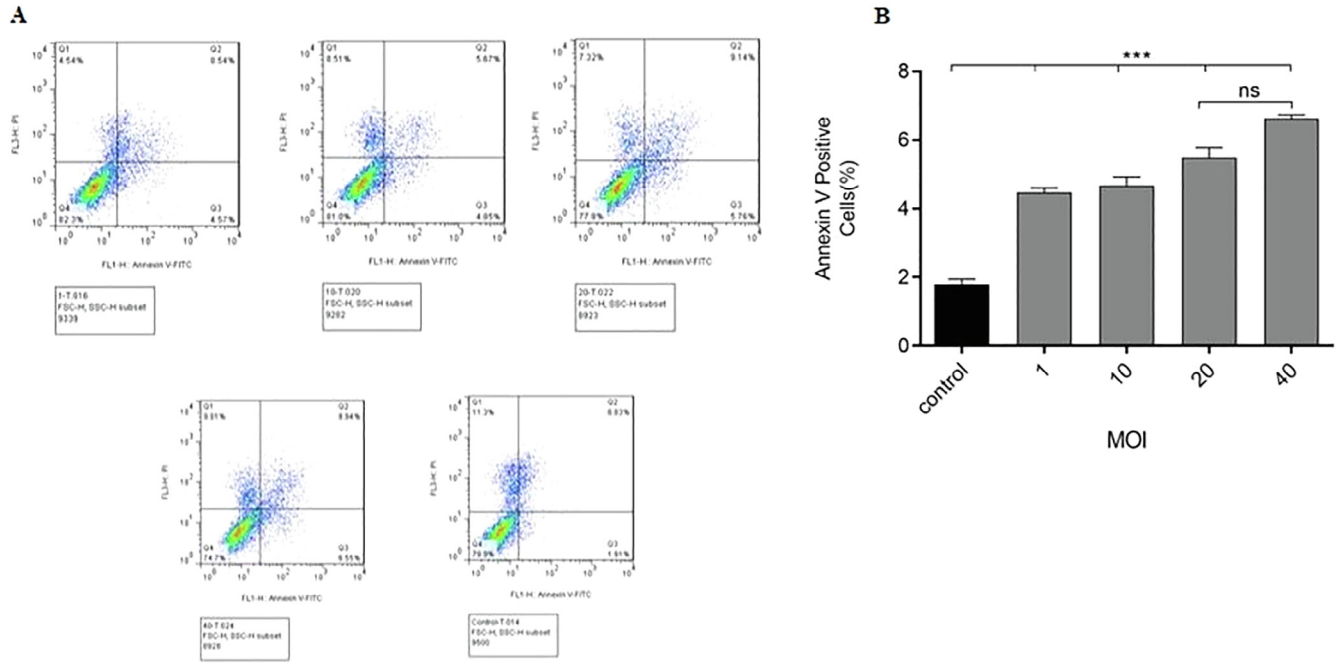

Fig. 3. Annexin V/PI staining of TC-1 cells treated with NDV. (A) TC-1 cells were treated with various MOI of NDV (1 to 40) for 72 h, after that expose to Annexin V/PI staining and analyzed via flow cytometry. Un-treated cells consider as control test. (B) The total percentage of apoptotic cells were stained with annexin V. The results showed statistically significant difference between all treatment groups (MOIs 1 to 40) compare to control. Results also indicates that no significant difference between MOI 20 compare to MOI 40 group ($p > 0.05$). $^{***}(P < 0.001)$ indicates statistically significant difference between all MOIs compared with control group by one-way ANOVA.

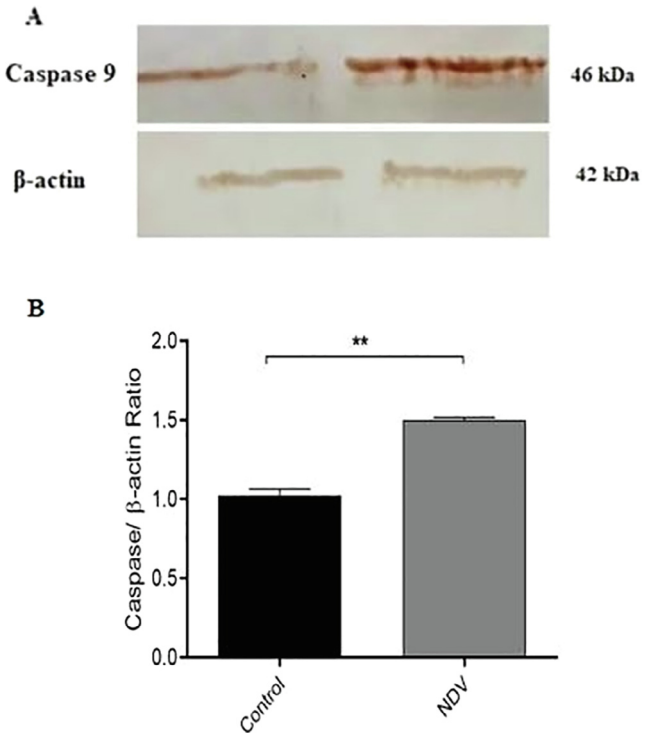

Fig. 4. (A) Representative western blotting of Caspase 9 protein after treatment with NDV (MOI: 20) in TC-1 cells. β-actin was used as a normalizer. (B) The protein levels of Caspase 9 in TC-1 cells after treatment with NDV (MOI: 20) and their negative control (un-infected cells). All the experiments were performed in triplicates. $^{**}(P < 0.01)$ indicates statistically significant difference between MOI 20 compared with control group by Student's *t*-test.

cell death in different cancer cells (Liou and Storz, 2010, Zhu et al., 2018). To further confirm apoptotic cell death caused by oncolytic NDV, we evaluated whether NDV-induced apoptosis in TC-1 cells was also related with enhanced ROS levels by quantifying intracellular ROS concentrations with DCFH-DA-based flow cytometry. For this, TC-1 cells were infected with NDV at the MOI of 20 as optimum oncolytic dose. As shown in Fig. 5, an increase in ROS generation was detected in NDV-induced TC-1 in a MOI -dependent manner than control cells ($p < 0.01$).

4. Discussion

The application of oncolytic virus is an attractive therapeutic strategy for cancer therapy. Oncolytic viruses are described as genetically engineered or naturally occurring strains that induce potent adaptive anti-tumor immunity via selectively infecting cancerous cells without harming the untransformed and normal tissues (Jhawar et al., 2017, Wei et al., 2018).

So far, a number of oncolytic viruses have demonstrated to be useful in pre-clinical studies and early clinical trials. Among them, NDV is known as a tremendously promising oncolytic agent due to its reasonable safety profile and associated cell death activating local and distant antitumor immune responses (Choi et al., 2016, Schirrmacher, 2015).

A number of clinical trials have been directed to use wild type NDV strains in cancer patients with various types of solid tumors with no considerable side effects (Sinkovics and Horvath, 2000). Thus, oncolytic NDV is considered an effective strategy for treating cancer.

The main objective of this study was to evaluate oncolytic efficacy of LaSota strain on TC-1 tumor cell line, its ability to destroy these cells in vitro. The murine TC-1 cells of human papillomavirus (HPV)-related carcinoma which express human papillomavirus 16 (HPV-16) E6/E7 oncoproteins were selected as a model system. For this purpose, TC-1 cell line was infected with LaSota strain, an oncolytic NDV, at MOI of 1 to 40 in vitro. To assess and determine oncolytic titer of NDV with regard to metabolic activity and cellular integrity, MTT tests and LDH release assays was performed respectively.

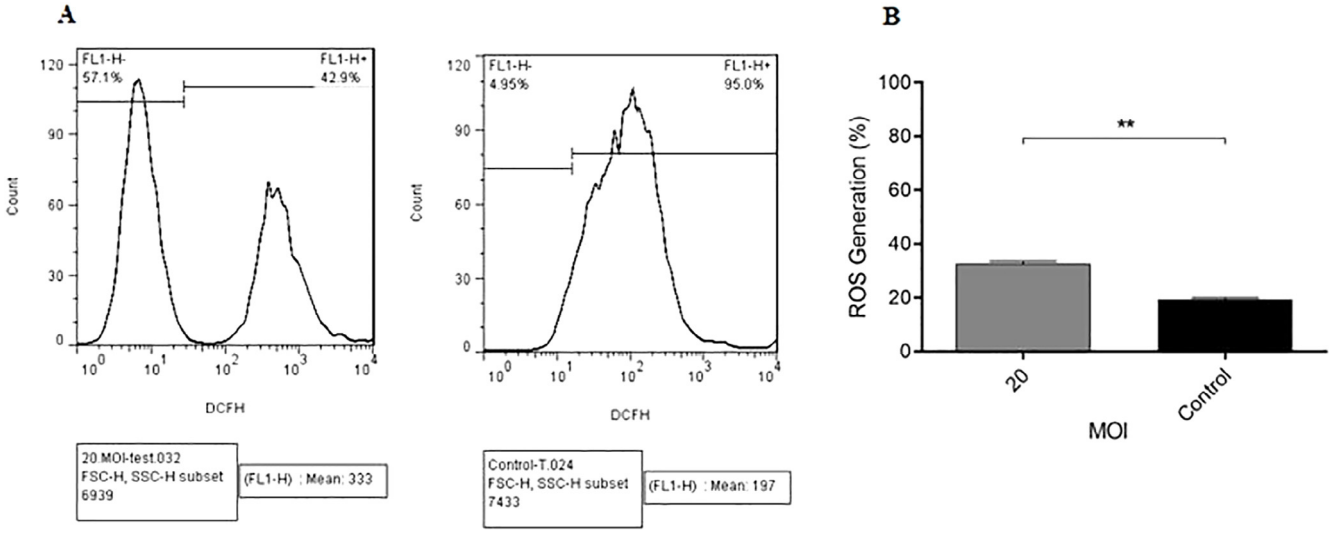

Fig. 5. DCFH-DA staining of TC-1 cells treated with NDV. (A) TC-1 cells were treated with NDV at MOI 20 for 72 h, Un-treated cells consider as control test. (B) The total percentage of apoptotic cells were stained with DCFH-DA. The results showed that infection with 20 MOI can significantly release high level of ROS vs control. $^{**}(P < 0.01)$ indicates statistically significant difference between MOI 20 compared with control group by Student's *t*-test.

The MTT assay determines the metabolic cells by measuring the amount of purple formazan produced by mitochondrial reduction of tetrazolium salts. While the leakage of cytoplasmic lactate dehydrogenase indicates release of the intracellular contents due to reduced membrane integrity—apoptotic or necrotic. To investigate the mechanism of its oncolytic and antitumor activity initially on HPV cancer cells, flow cytometry was used for detection of apoptotic cells by annexin V/PI staining and ROS measurement. Western blotting was to evaluate the levels of Caspase 9, an indicator of the cell apoptosis. Our results showed that NDV LaSota strain induced effective oncolytic activity against treated TC-1 cell line in a MOI-dependent manner. The results of MTT and LDH assays demonstrated significant MOI-dependent cell cytotoxicity of TC-1 cells with NDV after treatment. The oncolytic virus exerted its antitumor effect through apoptosis as evidenced by annexin V/PI double staining confirmed the engagement of apoptosis in cell death induction in MOI-dependent manner compared to control cells. Measuring the level of Caspase 9 in infected cells in comparison with the control showed that the NDV activated the internal pathway of the apoptosis. Moreover, results demonstrated that NDV LaSota strain leads to ROS accumulation in treated TC-1 tumor in a MOI- dependent manner in comparison with control cells. Increasing ROS levels can be resulted in cell death specially in cancer cells that contribute to the efficiency of NDV oncolysis.

Consistent with the current results, recent studies have reported the oncolytic effects of different wild-type NDV strains on the cell growth of several human cell lines without any side effect on healthy cells (Yurchenko et al., 2018). Krishnamurthy et al evaluated oncolytic effect of NDV on human fibrosarcoma and fibroblast cells. The results indicate that NDV rapidly replicated and destroy human fibrosarcoma cells (HT-1080) but it has no similar effect on the human skin fibroblast cells as control (CCD-1122Sk) (Krishnamurthy et al., 2006). In line with previous researches, another survey determined oncolytic activity of NDV on the human non-small-cell lung cancer cell line A549. The study revealed that NDV replication and syncytium formation increased in A549 tumor cells and leading to apoptosis formation (Mansour et al., 2011).

The mechanisms of oncolytic NDV-mediated destruction of the tumor cells have been studied in some recent experiments. The studies have reported that NDV can suppress growth of broad type of tumor cells through triggering of apoptosis via mitochondrial intrinsic pathway and the death receptor extrinsic pathway (Elankumaran et al., 2006, Ravindra et al., 2009).

The apoptosis induced by NDV in HeLa cells has been indicated to be mediated mostly by activation of TNF-related apoptosis-inducing ligand (TRAIL) and caspase pathways (Kumar et al., 2012). In addition, Ravindra et al. also found that mitochondrial intrinsic apoptotic pathway is a main mechanism of apoptosis induction in NDV-infected Vero cell line at 48 h post-infection (Ravindra et al., 2009). It has been shown that induction of cytopathic effects in NDV-infected cells is initiated by apoptosis that required viral replication and stimulation of caspase (Ravindra et al., 2008a). In these experiments, the contribution of the haemagglutinin neuraminidase (HN) protein in apoptosis triggered by NDV have been reported (Ravindra et al., 2008b).

ROS are mainly formed in mitochondria and are central contributors to different types of oxidative stress and cell death (Scherz-Shouval and Elazar, 2007). The high levels of ROS lead to cell death enhancing the cellular apoptotic pathway (Gao et al., 2016). ROS has been linked to autophagic cell death, a cellular process implicated with type II programmed cell death and described by development of autophagosomes (Levine and Klionsky, 2004). Autophagy has been associated in tumor suppression (Jin, 2005). Consistent with the current results, it has been demonstrated that secretion of ROS have resulted in apoptosis, mitochondrial impairment, and the caspases stimulation in oncolytic adenovirus–infected gastric adenocarcinoma cell (Wei et al., 2010).

5. Conclusion

In this study, the results reported herein provide evidence of the cytotoxic potential of a NDV vaccine strain LaSota towards TC-1 cell line of HPV-associated carcinoma, expressing human papillomavirus 16 (HPV-16) E6/E7 oncoproteins. It was demonstrated that this oncolytic virus induced antitumor activity in the cells via preferential replication in transformed cells, ROS production and activation of early apoptosis pathways. The data provide a novel insight in use of NDV in cervical cancer treatment.

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Conflict of interest

The authors declare that they have no conflict of interest.

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